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(54) Title: <b>INDUCTION OF B CELL TOLERANCE</b>  (57) Abstract  <p>The invention provides methods and compositions for promoting in a first species a state of tolerance against Gal<math>\alpha</math>1,3Gal epitopes present on a xenograft from a second species, thereby preventing hyperacute rejection (HAR) of the xenograft. The methods and compositions according to the invention cause the elimination or anergy of specific lymphoid cells which are responsible for the production of xenoreactive natural antibodies (XNAs) which cause HAR of the xenograft. In a first aspect, the invention provides methods and tolerogenic compositions for inducing anergy in B cells which are specific for the Gal<math>\alpha</math>1,3Gal epitope. In a second aspect, the invention provides methods and tolerogenic compositions for inducing apoptosis in B cells specific for the Gal<math>\alpha</math>1,3Gal epitope.</p>			

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## INDUCTION OF B CELL TOLERANCE

### BACKGROUND OF THE INVENTION

#### Field of the invention

The invention relates to induction of B cell tolerance. More particularly, the invention relates to induction of B cell tolerance to reduce or eliminate hyperacute rejection of xenogeneic organ transplants.

#### Summary of the related art

The increasing success in organ transplantation achieved in the past decade has resulted in a severe shortage of human donor organs. It is estimated that in the United States alone more than 33,000 people await organ transplants, whereas only about 5,000 organs are donated each year. One potential approach to closing this gap is xenogeneic organ transplantation.

Sachs, "MHC-homozygous miniature swine" in *Swine as Models in Biomedical Research*, Swindle *et al.* (eds.), Iowa State University Press, Ames, Iowa (1992), teaches that swine are the best studied organ donor species for xenotransplantation due to their size, availability and potential for genetic manipulation. Unfortunately, swine xenografts are rapidly rejected by primate hosts. Galili, *Immunol. Today* 14 : 480 (1993), teaches that the initial barrier to the transplantation of vascularized organs from pig to primate is hyperacute rejection (HAR), and that HAR is initiated by the deposition of xenoreactive natural antibodies (XNAs) onto the surface of porcine endothelial cells and subsequent activation of host complement results in destruction

of the graft by thrombosis, hemorrhage and edema.

Galili, *supra*, further teaches that the antigenic epitope recognized by human XNAs is the galactose  $\alpha$ 1,3 galactose epitope (Gal $\alpha$ 1,3Gal epitope). Galili *et al.*, *J. Exp. Med.* 160: 1519-1531 (1984), teaches that these anti-gal antibodies represent 1-4% of the total IgM and 1% of the total IgG in primates. Sandrin *et al.*, *Proc. Natl. Acad. Sci. USA.* 90: 11391-11395 (1993). teaches that it is the IgM XNAs, most of which are specific for Gal $\alpha$ 1,3Gal, which initiate the activation of human complement on porcine cells.

Thus, there is a need to remove these IgM XNAs to facilitate xenograft survival. Latinne *et al.*, *Transplant Proc.* 250: 336 (1993) teaches that removal of XNAs prolongs discordant xenograft survival and can be achieved through plasmapheresis, perfusion through a donor organ, or immunoadsorption techniques. Unfortunately, none of these techniques can permanently remove XNAs from a primate, so eventual rejection of the xenograft still occurs.

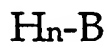
There is, therefore, a need for methods for permanently removing XNAs. One possible approach to permanent removal of XNAs is to induce a state of tolerance against the Gal $\alpha$ (1,3)Gal epitope using a tolerogen, which is an antigen which induces tolerance by inhibiting lymphocyte activation upon subsequent exposure to the antigen. Dintzis and Dintzis, *Proc. Natl. Acad. Sci. USA* 89: 1113-1117 (1992), teaches that ongoing T cell independent and T cell dependent immune responses can be immunosuppressed when an appropriate dose of antigen is administered. Conrad *et al.* (U.S. Patent Nos. 5,276,013 and 5,162,515) teaches a composition for treating the

autoimmune disease systemic lupus erythematosus comprising a chemically defined conjugate having a non-immunogenic backbone that is effective for inducing tolerance to autoantigens involved in lupus. Kiessling *et al.* (WO 96/20236) has described a polyglycomer with unique biological properties relative to monomeric ligands, but do not suggest a tolerizing effect. The challenge is to develop effective compositions and methods for inducing such tolerance to the Gal $\alpha$ 1,3Gal epitope.

### BRIEF SUMMARY OF THE INVENTION

The invention provides methods and compositions for promoting in an animal of a first species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second species, thereby preventing hyperacute rejection (HAR) of the xenograft. The methods and compositions according to the invention cause the elimination or anergy of specific lymphoid cells which are responsible for the production of xenoreactive natural antibodies (XNAs) which cause HAR of the xenograft.

In a first aspect, the invention provides methods and tolerogenic compositions for inducing anergy in B cells which are specific for the Gal $\alpha$ 1,3Gal epitope. Tolerogenic compositions according to this aspect of the invention comprise the structure



wherein H represents a Gal $\alpha$ 1,3 Gal epitope-containing hapten, B represents a backbone and - represents an operable linkage, and wherein n is a number from 1 to

100,000,000. In one preferred embodiment of a composition according to this aspect of the invention, H is a galactosyl galactose oligosaccharide having a terminal Gal $\alpha$ 1,3 Gal epitope. In a particularly preferred embodiment, H is a galactosyl galactose disaccharide or trisaccharide, most preferably Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc or Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc. The trisaccharide is most preferable. H preferably is not by itself immunogenic, but becomes immunogenic when linked to an immunogenic carrier molecule, such as a glycolipid or glycoprotein. According to this aspect of the invention, H becomes tolerogenic when linked to a backbone. The operable linkage includes any association between H and B. One preferred operable linkage is a covalent linkage between H and B. More preferably, the covalent linkage may be directly between H and B so as to integrate H into the backbone. Alternatively, the covalent linkage may be through an extended structure, preferably a non-immunogenic structure, such as an oligosaccharide, a glycolipid, autologous IgG, or an aliphatic hydrocarbon chain. Other preferred operable linkages include lipophilic association, such as formation of a liposome containing B and having H covalently linked to a lipophilic molecule and thus associated with the liposome. In a preferred embodiment, B is a non-immunogenic backbone, most preferably a non-immunogenic polymer having a molecular weight up to 100,000 Da, preferably from about 5,000 Da to about 100,000 Da. Particularly preferred non-immunogenic polymers include polyvinylethanolamines, polyacrylamides, dextrans and polyglycomers. In another preferred embodiment, B is a non-immunogenic cellular antigen. Particularly preferred non-immunogenic cellular antigens include autologous, syngeneic, or

xenogeneic erythrocytes. Tolerogenic compositions according to this aspect of the invention may also include pharmaceutically acceptable carriers, diluents, and/or controlled release agents. B may also represent an agent which is cytotoxic to or down modulates the function of T and B cells. Preferred agents, B, according to this aspect of the invention include without limitation, DMS, anti-IgM, lipophilic toxins, radioisotopes, diphtheria toxin and ricin A chain. In yet another aspect of the invention, B may be an immunogenic carrier, wherein the immunogenic carrier may include, without limitation, immunogenic proteins, lipids, carbohydrates, and cells such as pig cells and rabbit erythrocytes. Another preferred embodiment includes B incorporated in a liposome, such as a terminal  $\alpha$ galactosyl structures, or N,N-Dimethylsphingosine.

A method according to this first aspect of the invention promotes in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, thereby preventing HAR of the xenograft. The method according to this aspect of the invention comprises administering to an animal of the first species a tolerogenic amount of a tolerogenic composition according to this aspect of the invention. Such administration may be via the oral, intravenous, intramuscular, subcutaneous, intranasal, intradermal, or suppository routes, or by implanting.

In a second aspect, the invention provides methods and tolerogenic compositions for inducing apoptosis in B cells specific for the Gal $\alpha$ 1,3Gal epitope.

Tolerogenic compositions according to this aspect of the invention comprise

the structure



wherein H represents a Gal $\alpha$ 1,3 Gal epitope-containing hapten as described for the first aspect of the invention, T represents an agent which is cytotoxic to or down modulates the function of T and B cells and - represents an operable linkage, and wherein n is a number from 1 to 1,000.

Preferred agents, T, according to this aspect of the invention include without limitation, DMS, anti-IgM, lipophilic toxins, radioisotopes, diphtheria toxin and ricin A chain. The operable linkage includes any association between H and T which allows H to target T to B cells or T cells which are specific for the Gal $\alpha$ 1,3Gal epitope. One preferred operable linkage is a covalent linkage directly between H and T.

Alternatively, H and T may both be covalently linked to a carrier molecule. Other preferred operable linkages include lipophilic association, such as formation of a liposome containing T and having H covalently linked to a lipophilic molecule and thus associated with the liposome. In certain embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when H is associated with one liposome and T is associated with another liposome. For inducing apoptosis in T cells specific for H, any of the tolerogenic compositions according to this aspect may further be operably linked to a T cell receptor binding peptide or protein.

A method according to this second aspect of the invention promotes in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes



present on a xenograft from an animal of a second animal species, thereby preventing HAR of the xenograft. In a particularly preferred embodiment, the animal of the first animal species is a human and the animal of the second animal species is a pig. The method according to this aspect of the invention comprises administering to the animal of the first species a tolerogenic amount of a tolerogenic composition according to this aspect of the invention.

In a third aspect, the invention provides yet another method for promoting in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, thereby preventing HAR of the xenograft. In a particularly preferred embodiment, the animal of the first animal species is a human and the animal of the second animal species is a pig. The method according to this aspect of the invention comprises administering to the animal of the first species an immunogenic composition comprising the structure



wherein H represents a Gal $\alpha$ 1,3 Gal epitope-containing hapten as described for the first aspect of the invention, I represents an immunogenic carrier and - represents an operable linkage, and wherein n is a number from 1 to 100,000,000, then treating the animal with a chemotherapeutic agent. Exposure to the immunogenic composition causes memory B cells and immature B cells specific for H to differentiate and renders them sensitive to the chemotherapeutic agent. According to this aspect of the invention, H is as described for the first aspect of the invention, and the operable linkage is as described for the second aspect of the invention. Particularly preferred

immunogenic molecules, I, include without limitation immunogenic proteins, lipids, carbohydrates, and cells such as pig cells and rabbit erythrocytes. Particularly preferred chemotherapeutic agents include without limitation cyclophosphamide, leflunomide, mycophenolate mofetil, doxorubicin, 2,3-dideoxyinosine, chlorambucil, steroid hormones, adriamycin, brequinar analogs and melphalan.

For each of the methods described herein, depletion of plasma cells will preferably be carried out as well to eliminate resident plasma cells specific for the Gal $\alpha$ 1,3Gal epitope. Preferably, such plasma cell depletion will employ anti-CD38 immunotoxins, anti-HM1.24 antibodies, or radionuclide conjugates.

#### Brief Description of the Drawings

Figure 1 depicts the specificity of the anti-Gal ELISPOT assay using baboon spleen cells.

Figure 2 shows the specificity of the anti-Gal ELISPOT assay using GalT(-/-) mouse spleen cells.

Figure 3 reveals the Gal $\alpha$ 1,3 Gal XNA producing B cell response to treatment with  $\alpha$ 1,3Gal-BSA and cyclophosphamide. Figure 3A shows the IgM immune response and Figure 3B shows the IgG immune response.

Figure 4 establishes the Gal $\alpha$ 1,3 Gal XNA producing B cell response to treatment with cyclophosphamide alone. Figure 4A shows the IgM immune response and Figure 4B shows the IgG immune response.

Figure 5 demonstrates the effect of  $\alpha$ 1,3Gal-BSA injection followed by

cyclophosphamide on B cell response two weeks after pig PBMC challenge. MFI is the abbreviation for "mean fluorescence intensity" and CYP is the abbreviation for cyclophosphamide.

Figure 6 exhibits the effect of cyclophosphamide in the absence of  $\alpha$ 1,3Gal-BSA on B cell response to pig PMBC. MFI is the abbreviation for "mean fluorescence intensity" and CYP is the abbreviation for cyclophosphamide.

Figure 7 depicts the effect of  $\alpha$ Gal containing antigen pulses followed by metabolic inhibition on *in vivo* anti-Gal production in GalT(-/-) mice. Figure 7A shows the IgM immune response and Figure 7B shows the IgG immune response.

Figure 8 establishes that  $\alpha$ Gal and  $\alpha$ Gal /DMS containing liposomes completely inhibit *in vitro* anti-Gal production.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

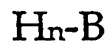
The invention relates to induction of B cell tolerance. More particularly, the invention relates to induction of B cell tolerance to reduce or eliminate hyperacute rejection (HAR) of xenogeneic organ transplants. The patents and publications cited herein illustrate the knowledge available to those skilled in this field and are hereby incorporated by reference in their entirety.

The invention provides methods and compositions for promoting in a first species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from a second species, thereby preventing HAR of the xenograft. The methods and compositions according to the invention cause the elimination or anergy of specific

lymphoid cells which are responsible for the production of the xenoreactive natural antibody (XNAs) which cause HAR. Such elimination or anergy is monitored by assessing the level of antibodies specific for the Gal $\alpha$ 1,3Gal epitope. Such elimination or anergy can further be monitored by improved survival of a transplanted xenogeneic organ or graft. The methods and compositions according to the invention are useful for promoting successful xenogeneic organ and cellular graft transplantation into human patients. In addition, the methods and compositions according to the invention are useful for promoting studies of xenogeneic organ and cellular graft transplants in non-human primates.

In a first aspect, the invention provides methods and tolerogenic compositions for inducing anergy in B cells which are specific for the Gal $\alpha$ 1,3Gal epitope. For purposes of the invention, the term "Gal $\alpha$ 1,3Gal epitope" refers to epitopes located wholly or partially on galactosyl ( $\alpha$ 1,3) galactose structures, such as the galactosyl ( $\alpha$ 1,3) galactose structure of  $\alpha$ Gal(1-3) $\beta$ Gal(1-4) $\beta$ GlcNAc or  $\alpha$ Gal(1-3) $\beta$ Gal(1-4) $\beta$ Glc structures.

Tolerogenic compositions according to this aspect of the invention comprise the structure



wherein H represents a Gal $\alpha$ 1,3Gal epitope-containing hapten, B represents a backbone and - represents an operable linkage and wherein n is a number from 1 to 1,000.

In one preferred embodiment of a composition according to this aspect of the invention, H is a galactosyl( $\alpha$ 1,3) galactose moiety having a terminal Gal $\alpha$ 1,3Gal epitope. In a particularly preferred embodiment H is a galactosyl( $\alpha$ 1,3)galactose disaccharide or trisaccharide, most preferably Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc or Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc. The most preferred embodiment is the trisaccharide. H preferably is not by itself immunogenic, but becomes immunogenic when linked to an immunogenic carrier molecule, such as a glycolipid or glycoprotein. According to this aspect of the invention, H becomes tolerogenic when linked to a backbone. The operable linkage includes any association between H and B. One preferred operable linkage is a covalent linkage between H and B. More preferably, the covalent linkage may be directly between H and B so as to integrate H into the backbone. Alternatively, it may be through an extended structure, preferably a non-immunogenic structure, such as an oligosaccharide, a glycolipid, autologous IgG, or an aliphatic hydrocarbon chain. Conjugation to the backbone can be achieved through the activation of H or the extended structure to which H is optionally attached, by an activating moiety, followed by coupling of the activated H to a functional group on the backbone. For example, when H is Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc, it can be activated by conjugation at the reducing end with 1-N glycyloisothiocyanate, then conjugated with a backbone containing amine groups. Other suitable activating moieties include, without limitation, 7-oxanonbornene. Other functional groups on the backbone which are suitable for conjugation with activated H include, without limitation, primary amines, hydroxyls, sulfhydryls and anhydrides. Other preferred

operable linkages include lipophilic association, such as formation of a liposome containing B and having H covalently linked to a lipophilic molecule and thus associated with the liposome. Accordingly, another preferred embodiment includes B incorporated in a liposome, such as a terminal  $\alpha$ galactosyl structures, or N,N-Dimethylsphingosine.

In a preferred embodiment, B is a non-immunogenic backbone, most preferably a non-immunogenic polymer having a molecular weight up to 100,000 Da, preferably from about 5,000 Da to about 100,000 Da. Particularly preferred non-immunogenic polymers include polyvinylethanolamines, polyacrylamides, dextrans and polyglycomers. Particularly preferred polyvinylethanolamines include those in the molecular weight range of about from 10,000 to about 14,000 Da. When B is such a polyvinylethanolamine, n is preferably 1-5, and most preferably 1.5-3. Particularly preferred polyacrylamides are polyacrylamide/amines in the molecular weight range of from about 40,000 to about 60,000 Da. When B is such a polyacrylamide/amine, n is preferably from about 6 to about 25, and most preferably from about 11 to 19. Particularly preferred dextrans include those in the molecular weight range of from about 60,000 to about 100,000 Da, with Dextran 70 being most preferred. When B is such a dextran, n is preferably from about 20 to about 40, and most preferably about 30. Particularly preferred polyglycomers are those in the molecular weight range of from about 5,000 to about 100,000 Da and include polyglycomers produced by polymerization of monomeric compounds containing divalent  $\text{Gal}\alpha 1,3\text{Gal}\beta\text{D}1,4\text{GlcNAc}$  spaced at regular intervals. Suitable monomers for such

polymerization include, without limitation, 7-oxanobornene-H. When B is such a polyglycomer, n is preferably from about 20 to about 40, and most preferably about 30.

Particularly preferred non-immunogenic cellular antigens include autologous, syngeneic, or xenogeneic erythrocytes. Such cellular antigens may be conjugated to H in a manner similar to that described for the non-immunogenic polymers described above, for example by conjugation to epsilon amino groups from cell surface proteins. Alternatively, when such cellular antigens are autologous erythrocytes or xenogeneic erythrocytes which do not express Gal $\alpha$ 1,3Gal epitopes, they can be made to express such epitopes either enzymatically or by transfection of an  $\alpha$ 1,3galactosyl transferase cDNA or mRNA. Other suitable cellular antigens include, without limitation, any syngeneic cell linked to H. When B is such a cellular antigen, n is preferably from about 100,000 to 100,000,000, and most preferably from about 1,000,000 to about 10,000,000. For purposes of embodiments of the invention in which B is such a cellular antigen, the term "covalent linkage" is intended to encompass lipophilic association. Lipophilic association includes the formation of a liposome containing T and having H covalently linked to a lipophile and thus associated with the liposome.

Generally, suitable backbone, B, for the tolerogenic compositions according to this aspect of the invention can be identified according to the following criteria. First, they are preferably non-immunogenic when administered to a primate, including a human. As a practical matter, this can be evaluated by determining the level of antibodies specific for a candidate backbone molecule in peripheral blood of a

primate, including a human, prior to administration of the candidate backbone molecule, followed by administration of the candidate backbone molecule to the primate, assessment of the post-administration level of antibodies specific for the candidate backbone molecule, and comparison of the level of such antibodies to the level of such antibodies prior to administration of the candidate backbone molecule. Suitable candidates will not cause a significant increase in the level of antibodies to the candidate backbone molecule. Second, suitable non-immunogenic backbones should contain suitable functional groups for conjugation with the activated H. As a practical matter, this can be assessed by activating the H molecule, attempting conjugation to B, and determining whether such conjugation has been successful. Such determination can be carried out by procedures, such as radiolabeling of H and post-conjugation assessment of the molecular weight of the radioactive species present in the conjugation reaction mixture. Third, the non-immunogenic backbone should be sufficiently non-toxic to allow its safe administration to the primate, including a human. Finally, when conjugated to H, the non-immunogenic backbone should form a complex which induces in a primate tolerance to the Gal $\alpha$ 1,3Gal epitope, as described below.

Alternatively, B may also represent an agent which is cytotoxic to or down modulates the function of T and B cells. Preferred agents, B, according to this aspect of the invention include without limitation, DMS, anti-IgM, lipophilic toxins, radioisotopes, diphtheria toxin and ricin A chain. In yet another aspect of the invention, B may be an immunogenic carrier, wherein the immunogenic carrier may



include, without limitation, immunogenic proteins, lipids, carbohydrates, and cells such as pig cells and rabbit erythrocytes.

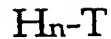
Tolerogenic compositions according to this aspect of the invention may also include pharmaceutically acceptable carriers, diluents, and/or controlled release agents. Certain non-limiting examples of such carriers, diluents and/or controlled release agents include buffered saline, oils, implantable pumps and encapsulated beads.

A method according to this first aspect of the invention promotes in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, thereby preventing HAR of the xenograft. In a particularly preferred embodiment, the animal of the first animal species is a human and the animal of the second animal species is a pig. The method according to this aspect of the invention comprises administering to the animal of the first species a tolerogenic amount of a tolerogenic composition according to this aspect of the invention. Such administration may be via the oral, intravenous, intramuscular, subcutaneous, intranasal, intradermal, or suppository routes, or by implanting. For initial administration, dosage will depend in part on the size and hapten density of the particular tolerogenic composition used. Generally, initial dosing will preferably be in the range of about 0.1 to 10 g per 25 kg body weight, and most preferably about 1 g/25 kg. The dosage regimen may be adjusted based upon the response achieved in a particular subject individual. For example, in certain cases a single injection might be sufficient to induce tolerance. In other cases, several

divided doses may be administered daily or the dose may be proportionally reduced as onset of tolerance is observed. Residual non-tolerized Gal $\alpha$ 1,3Gal epitope-specific cells can be eliminated by administration of chemotherapeutic agents, as described for the third aspect of the invention. Tolerance induction is preferably monitored by standard immunoassays for the presence of antibodies specific for the Gal $\alpha$ 1,3Gal epitope. Specifically, an ELISPOT assay is performed to determine the frequency of  $\alpha$ Gal secreting B cells. As used herein, "tolerance" refers to a reduction in the immune response specific for Gal $\alpha$ 1,3Gal epitope to a level that is lower than it would have been had the method according to the invention not been employed. Such immune response reduction can be humoral, cellular or both. Such tolerance can be measured, for example, by quantitating a reduction in antibodies specific for Gal $\alpha$ 1,3Gal epitope. Preferably, such tolerance will lead to a reduction in such antibodies by 90% or more, and most preferably by 99% to 100%. Tolerance can be confirmed by xenogeneic transplantation, *e.g.*, xenogeneic bone marrow transplantation. Once such tolerance is achieved, it can be maintained by the continuous presence of tissue-associated or soluble Gal $\alpha$ 1,3Gal epitopes, either in the form of an organ graft, a cellular graft, or by repeated tolerization at regular intervals with cellular and/or soluble tolerogens.

In a second aspect, the invention provides methods and tolerogenic compositions for inducing apoptosis in B cells specific for the Gal $\alpha$ 1,3Gal epitope.

Tolerogenic compositions according to this aspect of the invention comprise the structure



wherein H represents a Gal $\alpha$ 1,3 Gal epitope-containing hapten as described for the first aspect of the invention, T represents an agent which is cytotoxic to or down modulates the function of T and B cells and - represents an operable linkage, and wherein n is a number from 1 to 1,000.

Preferred cytotoxic agents, T, according to this aspect of the invention include without limitation ceramide, anti IgM, lipophilic toxins such as DMS, TMS, ceramide analogs, radioisotopes such as a radioisotope of Tc,  $^{131}\text{I}$ ,  $^{90}\text{Yt}$  diptheria toxin, saponin, ricin A chain and doxorubicin. The operable linkage includes any association between H and T which allows H to target T to B cells or T cells which are specific for the Gal $\alpha$ 1,3Gal epitope. One preferred operable linkage is a covalent linkage directly between H and T. Linkages of this type may be formed by activating H and coupling the activated H to an appropriate functional group on T. Alternatively, H can be indirectly covalently linked to T through coupling of both H and T to a carrier molecule. For this embodiment, preferred carrier molecules include without limitation neoglycoproteins, such as bovine serum albumin or human serum albumin, other glycoproteins, such as immunoglobulins, interleukins, B cell receptor binding molecules and peptides derived from such neoglycoproteins or glycoproteins, as well as glycolipids, such as HDPE and ceramide, and synthetic carriers, such as polyglycomers. Both the direct and indirect types of covalent linkages between H and T can be obtained in the manner described for the compositions according to the first aspect of the invention.

Other preferred operable linkages include lipophilic association, such as formation of a liposome containing T and having H covalently linked to a lipophilic molecule and thus associated with the liposome. Such lipophilic molecules include without limitation phosphatidylcholine, cholesterol and phosphatidylethanolamine, and synthetic neoglycolipids, such as sialyllacNAc-HDPE. In one particularly preferred embodiment having lipophilic association as the operable linkage, T is a ceramide, such as ceramide pentahexoside. This embodiment may optionally further contain an inhibitor of PKC and/or Bcl-2, such as a sphingosine, and most preferably N,N-dimethylsphingosine (DMS) or N,N,N-trimethylsphingosine (TMS), to promote apoptosis when PKC is operational. In certain preferred embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when H is associated with one liposome and T is associated with another liposome. For inducing apoptosis in T cells specific for H, any of the tolerogenic compositions according to this aspect may further be operably linked to a T cell receptor binding peptide or protein.

This second aspect of the invention also provides a method for promoting in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, thereby preventing HAR of the xenograft. In a particularly preferred embodiment, the animal of the first animal species is a human and the animal of the second animal species is a pig. The method according to this aspect of the invention comprises administering to the animal of the first species a tolerogenic amount of a tolerogenic composition

according to this aspect of the invention. Administration, dosage and monitoring are all as described for the method according to the first aspect of the invention.

In a third aspect, the invention provides yet another method for promoting in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, thereby preventing HAR of the xenograft. In a particularly preferred embodiment, the animal of the first animal species is a human and the animal of the second animal species is a pig. The method according to this aspect of the invention comprises administering to the animal of the first species an immunogenic composition comprising the structure



wherein H represents a Gal $\alpha$ 1,3 Gal epitope-containing hapten as described for the first aspect of the invention, I represents an immunogenic carrier and - represents an operable linkage, and wherein n is a number from 1 to 10,000, then treating the animal with a chemotherapeutic agent. Exposure to the immunogenic composition causes memory B cells and immature B cells specific for H to differentiate and renders them sensitive to the chemotherapeutic agent. According to this aspect of the invention, H is as described for the first aspect of the invention, and the operable linkage is as described for the second aspect of the invention. Particularly preferred immunogenic molecules, I, include without limitation proteins, such as BSA, KLH, cholera toxin B subunit and polymers having a molecular weight over 100,000 Da, as well as immunogenic lipids, such as rabbit erythrocyte membranes, and immunogenic carbohydrates, such as dextran polymers having a molecular weight

over 100,000 Da.

Preferred chemotherapeutic agents include without limitation cyclophosphamide, leflunomide, mycophenolate mofetil, doxorubicin, 2,3-dideoxyinosine, chlorambucil, steroid hormones, adriamycin, brequinar analogs and melphalan. Preferably, the immunogenic composition according to this aspect of the invention is administered in the presence of adjuvant, or with xenogeneic lymphocytes or bone marrow cells in the absence of adjuvant. Preferred adjuvants include without limitation alum, RIBI and complete Freund's adjuvant. Preferred xenogeneic lymphocytes or bone marrow cells are those from an animal of the second animal species.

For each of the methods described herein, depletion of plasma cells will preferably be carried out as well to eliminate resident plasma cells specific for the Gal $\alpha$ 1,3Gal epitope. Preferably, such plasma cell depletion will employ anti-CD38 immunotoxins, radionuclide conjugates, or anti-HM1.24 antibodies. Preferred anti-CD38 immunotoxins include without limitation ricin A chain, diphtheria toxin A chain and saponin. Preferred radionuclide conjugates include without limitation a radioisotope of Tc,  $^{131}\text{I}$ ,  $^{90}\text{Yt}$ .

In certain additional preferred embodiments:

The method further includes introducing a graft from a donor mammal into the recipient mammal. The donor can be of a second species, e.g., a species which normally expresses the Gal $\alpha$ (1,3)Gal moiety, a species which expresses  $\alpha$ 1,3 galactosyltransferase activity, e.g., a swine, e.g., a miniature swine.

The method includes depleting XNAs from the blood of the recipient, e.g., by hemoperfusing an organ, e.g., a liver or kidney, obtained from a mammal of the donor species or by contacting the blood of the recipient with galactosyl $\alpha$ (1,3) galactose moieties coupled to an insoluble substrate, administering drugs which inhibit activation of B cells expressing XNAs (e.g., deoxyspergualin, DSG, Bristol) or administering to the recipient anti-IgM antibodies.

Furthermore, the method features a method of inducing tolerance in a recipient mammal of a first species to a graft from a donor mammal of a second species. The method includes: introducing, e.g., by intravenous injection, into the recipient mammal, hematopoietic stem cells; (optionally) inactivating the natural killer cells of the recipient mammal and preferably implanting the graft into the recipient. The hematopoietic cells are believed to prepare the recipient for the graft that follows, by inducing or maintaining tolerance at both the B cell and the T cell levels.

The method includes the creation of hematopoietic space to enable the introduced hematopoietic stems to engraft in the recipient. If needed the creation of hematopoietic space can be achieved by the administration of antibodies or drugs which deplete the bone marrow, e.g., by administering an inhibitor of cell proliferation, e.g., DSG, or an anti-metabolite, e.g., Brequinar, or an anti-T cell antibody, e.g., one or both of an anti-CD4 or anti-CD8 antibody. Hematopoietic space can also be created by irradiating the recipient mammal with low dose, e.g., between about 100 and 400 rads, whole body irradiation to deplete or partially deplete the bone marrow of the recipient. The creation of hematopoietic space does

not totally ablate the recipients bone marrow but allows for the production of mixed chimerism. The need for hematopoietic space can be minimized by the creation in the recipient of thymic space.

Other preferred embodiments include: the step of creating thymic space in the recipient, e.g., by irradiating the thymus of the recipient, e.g., by administering between 100 and 1,000, more preferably between 300 and 700, e.g., 700 rads, of thymic irradiation, or by administering anti-T cell antibodies in sufficient dose to inactivate thymocytes. Other methods for the creation of thymic space include: the administration of steroids, corticosteroids, Brequinar, or immune suppressant drugs, e.g., rapamycin, cyclosporin, or FK506. Methods of creating thymic space are disclosed in provisional U.S. Application Number 60/017,099 hereby incorporated by reference. The methods disclosed herein can be combined with the methods disclosed in provisional U.S. Application Number 60/017,099.

In other preferred embodiments, the method includes: inactivating T cells of the recipient mammal, e.g., by prior to introducing recipient cells or a graft into the recipient mammal, introducing into the recipient mammal an antibody capable of binding to T cells of the recipient mammal.

In preferred embodiments, the method includes: inactivating the natural killer cells of the recipient mammal, e.g., by prior to introducing the cells or a graft into the recipient mammal, introducing into the recipient mammal an antibody capable of binding to natural killer cells of the recipient mammal.



One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum. As is discussed below, preferably, a second anti-mature T cell antibody can be administered as well, which lyses T cells as well as NK cells. Lysing T cells is advantageous for both bone marrow and xenograft survival. Anti-T cell antibodies are present, along with anti-NK antibodies, in anti-thymocyte anti-serum. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

The methods described herein can be combined with methods of inducing tolerance described in U.S. Serial Number 08/266,427, filed June 27, 1994, the contents of which are hereby expressly incorporated by reference. Thus, the methods disclosed herein can include administering to the recipient a recipient cell which expresses a donor MHC class I gene or a donor MHC class II gene (or both). The cell which expresses the donor MHC gene can be the same cell which expresses the galactose  $\alpha$ 1,3 galactose moiety or it can be a different cell.

In preferred embodiments, a short course of help reducing treatment can be used to induce tolerance to the recipient cell or the graft. In particular, the methods described in U.S. Serial Number 08/458,720, filed June 1, 1995, the contents of which are expressly incorporated herein by reference, can be combined with the methods described herein.

In preferred embodiments, a short course of an immunosuppressive agent can be administered to inhibit T cell activity in the recipient. In particular, the methods described in U.S. Serial Number 08/458,720, filed June 1, 1995, the contents of which

are expressly incorporated herein by reference, can be combined with the methods described herein.

Methods of inducing tolerance by the methods described herein can also be combined with yet other methods for inducing tolerance, e.g., with: methods which use the implantation of donor stem cells to induce tolerance, e.g., the methods described in United States Serial No. 08/451,210, filed on May 26, 1995, the contents of which are hereby expressly incorporated by reference; methods which use stem cells or other tissue from genetically engineered swine, e.g., the genetically engineered swine in United States Serial No. 08/292,565, filed August 19, 1994, the contents of which are expressly incorporated herein by reference, or in United States Serial No. 08/692,843, filed August 2, 1996, the contents of which are expressly incorporated herein by reference; methods which use the implantation of a xenogeneic thymic graft to induce tolerance, e.g., the methods described in United States Serial No. 08/163,912, filed on December 7, 1993, the contents of which are hereby expressly incorporated by reference; methods of increasing the level of the activity of a tolerance promoting or GVHD inhibiting cytokine or decreasing the level of activity of a tolerance inhibiting or GVHD promoting cytokine, e.g., the methods described in United States Serial No. 08/114,072, filed August 30, 1993, the contents of which are hereby expressly incorporated by reference; methods of using cord blood cells to induce tolerance, e.g., the methods described in United States Serial No. 08/150,739 filed November 10, 1993, the contents of which are hereby expressly incorporated by reference; methods of preventing GVHD, e.g., the methods described

in United States Serial No. 08/461,693, filed June 5, 1995, the contents of which are hereby expressly incorporated by reference; with methods of promoting tolerance by enhancing or maintaining thymus function, e.g., the methods described in United States Serial No. 08/297,291, filed August 26, 1994, the contents of which are hereby expressly incorporated by reference; methods of detecting the presence of swine retroviral sequences, e.g., the methods described in United States Serial No. 08/572,645, filed December 14, 1995, the contents of which are hereby expressly incorporated by reference; and the methods for inducing tolerance disclosed in Sykes and Sachs, PCT/US94/01616, filed February 14, 1994, the contents of which are hereby expressly incorporated by reference.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature.

#### Example 1

##### Synthesis of polyglycomers containing polyvalent Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc

Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc trisaccharides are prepared from GlcNAc and Gal monomers.  $\alpha$ -D-GlcNAc tetraacetate is purchased from Alberta Research Council (Edmonton, Alberta, Canada). The protected GlcNAc (1 eq.) is dissolved in dry acetonitrile and cooled to 4°C under nitrogen. Allyltrimethylsilane (3.5 ml, 3 eq.), freshly distilled borontrifluoride etherate (1.8 ml, 2 eq.) and trimethylsilyl triflate (0.56 ml, 0.4 eq.) are added sequentially. After stirring at 4°C for 2 hours, followed by 2 hours at 20°C, the mixture is cooled to 4°C and excess acid is quenched with 12 ml

of aqueous sodium bicarbonate. The solution is concentrated by rotary evaporation and extracted 3x with 75 ml  $\text{CH}_2\text{Cl}_2$ . The combined organic phases are dried and evaporated. The residue is then purified by flash chromatography. The resulting 1-C-allylGlcNAc is deacylated by reaction with NaOH (2 mg/ml) in methanol. The residue is filtered through a plug of silica gel. The product (1 eq.) is dissolved in 30 ml distilled pyridine with gentle warming and cooled to  $4^\circ\text{C}$  under argon. Triethylsilyl triflate (8 eq.) is added dropwise. The mixture is stirred for 8 hours at room temperature, then diluted with ether (200 ml) and allowed to separate into phases. The ether phase is washed 3x with aqueous ammonium chloride, then dried and evaporated. The residue is purified by flash chromatography to yield 1-C-allyl-3,4,5-tetra-O-triethylsilyl- $\beta$ -D-GlcNAc. This product (1.5 mmol) is dissolved in 12 ml of 1:2  $\text{CH}_2\text{Cl}_2$ /MeOH and cooled to  $-78^\circ\text{C}$ . Ozone is bubbled through the solution for 6 minutes until the solution is saturated with ozone. Sodium borohydride (15 mmol) is added and the mixture is stirred at  $-78^\circ\text{C}$  for one hour, then at  $3^\circ\text{C}$  for one hour. The reaction is quenched with aqueous ammonium chloride (15 ml), then stirred for one hour at  $3^\circ\text{C}$ . 20 ml diethyl ether is added and the aqueous layer is extracted 3x with 15 ml ether. The combined ether extracts are washed with brine, dried over  $\text{MgSO}_4$  and evaporated. The crude alcohol product, 1-C-EtOH-3,4,5-tetra-O-triethylsilyl- $\beta$ -D-GlcNAc, is purified by flash chromatography.

The 4-C-OH-1,2,3,5-tetra-O-triethylsilyl- $\beta$ -D-Gal alcohol is similarly prepared, except that the starting material is  $\alpha$ -D-Gal-1,2,3,5- tetraacetate. The alcohol products are separately co-distilled with toluene to remove water.

The 3-C-OH-1,2,4,5-tetra-O-triethylsilyl- $\beta$ -D-Gal alcohol is similarly prepared, except that the starting material is  $\beta$ -D-Gal-1,2,4,5-tetraacetate. The 1-OH disaccharide is generated with sodium borohydride, as described previously. The trisaccharide is generated by condensation between the Gal and the disaccharide. The trisaccharide alcohol is purified by flash chromatography.

3,6-oxy-1,2,3,6-tetrahydrophthalic anhydride (1 eq.), 4-dimethylaminopyridine (0.4 eq.) and 2-chloro-1-methylpyridinium iodide (1.2 eq.) are then added to 1 eq. of the trisaccharide alcohol. The flask is filled with argon and 2.0 ml  $\text{CH}_2\text{Cl}_2$  is added, followed by tripropylamine (3 eq.), then stirred at 22°C overnight to form a solution. The solution is then diluted with 20 ml ether and the ether layer is washed 2x with 10 ml aqueous ammonium chloride and 2x with 10 ml brine, then dried over  $\text{MgSO}_4$  and evaporated. The TES-protected trisaccharide-substituted monomer is purified by flash chromatography, then azeotroped with toluene and dissolved in 7.5 ml THF. The resulting solution is cooled in an ice bath, then 1 ml HF-pyridine is added dropwise. The resulting mixture is stirred at 0°C for 1.5 hours, then concentrated under reduced pressure. The residue is purified by flash chromatography. The resultant 7-oxanobornene is polymerized in  $\text{RuCl}_3 \cdot \text{H}_2\text{O}$  under nitrogen gas in degassed water, which is heated at 55-60°C. After 18 hours, the gel product is washed with acetone, then methanol to afford a solid, which is then dissolved in 10 ml water, concentrated to 2 ml and precipitated with 10 ml methanol. Excess solvent is then evaporated under reduced pressure.

### Example 2

#### Synthesis of dextrans conjugated to Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc

Dextran 70 is purchased from Sigma (St. Louis, MO), then converted to a polyamine by cyanogen bromide. Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc trisaccharides are synthesized as described in Example 1, then imidiazolated by reaction with N,N'-carbonyldiimidazole. Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-imidazole is then conjugated to the Dextran 70 polyamine by incubation in pH 5.0 PBS at 4°C. To obtain about 20-40 Gal $\alpha$ 1,3Gal epitopes per molecule, a molar ratio of 30:1 is used.

### Example 3

#### Preparation of a Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc/ceramide/sphingosine liposome

Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-lipid (HDPE) is purchased from Dextra Labs (Reading, UK). Ceramide pentahexoside is purified from rabbit erythrocyte membranes by extraction with chloroform/methanol (2:1, then 1:1, then 1:2), followed by RPLC on a 7.5 ml C18 column using chloroform/methanol (1:1) as eluant. One ml fractions are taken and monitored by TLC stained with orcinol.

N,N-dimethylsphingosine (DMS) is purchased from Calbiochem La Jolla, CA).

These molecules are formulated into liposomes having about 0.5%-25%

Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-lipid by molarity, 0.5% to 10% ceramide pentahexoside by molarity and 0.5% to 10% DMS by molarity. This is accomplished by lyophilization followed by freeze-thaw and high pressure or dialysis liposome formation.

#### Example 4

##### Preparation of a polyvinylethanolamine-Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc conjugate

Polyvinylethanolamine (PVE, Mr = 10,000-14,000) was purchased from Sigma. Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc was activated as described in Example 2. PVE and the activated trisaccharide were then incubated at a molar ratio of 1:2 to 1:10 for 1-2 hours at a temperature of 20-37°C to produce a conjugate having about 1.5-3 Gal $\alpha$ 1,3Gal epitopes per molecule.

#### Example 5

##### Development of an ELISPOT assay to determine the frequency of anti-Gal secreting B cells

In order to determine how experimental manipulations aimed at tolerance induction affect production of anti-Gal, an ELISPOT assay was developed to detect anti-Gal antibody production by B cells in an overnight culture *in vitro*. This assay was based on the detection of binding of antibodies secreted by individual cells to synthetic neoglycoprotein-coated microwells. The method essentially involved the isolation of lymphocytes or lymphocyte preparations enriched for B cells from various tissues. These cells were incubated in microwells in a hybridoma culture medium containing insulin, transferrin, and selenium, with or without fetal calf serum. In some cases, cells were immediately transferred to nitrocellulose bottom microtiter wells coated with antigen. Alternatively, cells were allowed to incubate with different cytokines and/or antibodies for up to three days before adding them

to the antigen coated microwells. After an overnight incubation in the antigen coated microwells, cells were washed from the wells and goat anti-mouse IgM or IgG conjugated to horseradish peroxidase was added. After incubation and removal of the secondary reagent, a peroxidase substrate was added. Spot formation was then taken to represent clonal secretion of antigen specific antibody. Each antigen specific clone is represented as a spot forming unit (SFU). The frequency of cells secreting antibody is then calculated as the number per  $10^5$  cells added to the microwells. The GalT(-/-) mouse was used throughout these studies as a model for the Gal $\alpha$ 1,3Gal specific XNA in a murine model. It is an embryonic stem cell knockout of the Galactosyltransferase gene. The result is a mouse which produces Gal $\alpha$ 1,3Gal specific XNA.

Specifically, the methodology was as follows:

A microtiter plate (Millipore 96-well filtration plate, 0.45  $\mu$ m surfactant-free mixed cellulose Ester membrane, Qyt: 10/pack, Cat# MAHAS4510) was aseptically coated with 100ul/well of 5ug/ml  $\alpha$ Gal-BSA in 1xPBS, and/or 100ul/well of unlabeled IgM and IgG at 5ug/ml in 1xPBS. Appropriate controls, such as N-acetyllactosamine-BSA were included. The coated plates were incubated overnight at 4°C, or 2 hours at 37°C. After incubation, the antigen solution was aseptically pipetted out of the plates. The plates were then washed by pipetting 200ul/well of 1xPBS, sitting for 5 minutes and then pipetting out of the solution. The wells were washed two times with 200ul/well of PBS and pipetted with 1xPBS up and down. Next, the microtiter wells were blocked for non-specific antibody binding with



200ul/well of IMDM (Iscove's Modified Dulbecco's Medium) supplemented with 0.4% BSA and 1ml/500ml of Gentamicin and incubated for one hour at 37°C. The blocking medium was removed by pipetting it out. Then, 200ul/well of cell culture medium was added to rows B-D and rows F-H. The cell culturing medium was made with IMDM supplemented with 10% Fetal Bovine Serum. Then, 250ul/well of the spleen cell preparation with a concentration of  $4 \times 10^6$  cells per ml was added to each well. The cells were serially diluted by 1/5 by taking 50ul from row A and transferring it to row B and then removing 50ul from row B and transferring it to row C and the same for row C to row D. 50ul was removed from row D and discarded to have 200ul in each well. This dilution was repeated with rows E through H. The plates were incubated overnight at 37°C, with 5% CO<sub>2</sub>. Following incubation, the plates were emptied by dumping and "flicking" out. The plates were washed three times with 200ul/well of 1xPBS. For the first two washes, 1xPBS was pipetted up and down to get rid of cells stuck to the membrane and to reduce the background. Then the plates were washed three times with 200ul/well of 1xPBS + 0.1% Tween-20. Next, 10ul/well of HRP-conjugated anti-mouse IgG or IgM was added and the wells were diluted to 1/1000 in 1xPBS supplemented with 0.5% Tween, and 0.4% BSA. The plates were then emptied out by dumping and "flicking" out the solution. The plates were washed three times with 200ul/well of 1xPBS + 0.1% Tween-20 followed by three washes with 200ul/well of 1xPBS. The substrate solution was added at 100ul/well and incubated at room temperature for 30 minutes. The substrate was made by dissolving 1 AEC (3-amino-9-ethylcarbazole) tablet

(Sigma A-6926) in 2.5ml Dimethylformamide. After it has dissolved, 47.5ml of 50mM Acetate buffer, pH 5.0 (74ml of 0.2N acetic acid, and 176ml 0.2M sodium acetate, then deionized water up to 1000ml) was added. 25ul of fresh 3% H<sub>2</sub>O<sub>2</sub> was added just before adding the substrate solution to the plate. The reaction was stopped by running tap water over the plate and dumping/flicking the water in the wells into the sink, and blotting dry. The bottom plastic section of the plate was removed and placed on C fold towels. The plate was wrapped in aluminum foil and allowed to dry at room temperature for 1-2 days. Spots were visualized with a stereomicroscope and dissecting with vertical white light. The spots were counted as the number of dark centers with rings of diffusion per well. The wells were averaged in triplicate, and divided by 8 to get SFUs (Spot Forming Units) per 10<sup>5</sup> cells. For example,

$$4.0 \times 10^6 \text{ cells/ml} \times 0.2 \text{ ml/well} = 8.0 \times 10^5 \text{ cells/well}$$

Diluted by 1/5	= 1.6x10 <sup>5</sup> cells/well
Diluted by 1/5	= 3.2x10 <sup>4</sup> cells/well
Diluted by 1/5	= 6.4x10 <sup>3</sup> cells/well

The specificity of the anti-Gal ELISPOT assay for Gal $\alpha$ 1,3Gal epitopes was demonstrated by incubating either GalT(-/-) spleen cells or frozen baboon spleen cells overnight in plates coated with terminal Gal $\alpha$ 1,3Gal, Lacto-N-fucopentaose (LNFP) or N-Acetyllactosamine (LacNAc) conjugated to BSA. Specific spot formation was only significantly above background when baboon spleen cells were cultured on Gal $\alpha$ 1,3Gal-linked neoglycoprotein (Figure 1). The specificity of the anti-Gal ELISPOT analysis using GalT(-/-) mouse spleen cells was also shown in an

analogous manner to that of the baboon spleen ELISPOT data (Figure 2).

The results of these experiments indicated that this ELISPOT assay is highly specific for the detection of the frequency of anti-Gal secreting plasma cells. Therefore, this ELISPOT assay has been used as the basis for the determination of the state of anti-Gal production by B cells in many subsequent experiments.

#### Example 6

##### Induction of B cell hyporesponsiveness created by antigenic stimulation followed by metabolic inhibition

The protocols that are currently used to deplete anti-Gal $\alpha$ 1, 3Gal antibodies from sera do not affect the production of these natural antibodies. Since porcine bone marrow cells express the Gal $\alpha$ ,3Gal epitopes, it is felt that the continuous production of these antibodies is detrimental to bone marrow engraftment and survival. One theory which might explain why the production of anti-Gal $\alpha$ 1,3Gal antibodies is refractory to the standard conditioning regimen is that, at the time of conditioning, only a proportion of B cells capable of activation by Gal $\alpha$ 1,3Gal epitopes are stimulated. The resting B cells therefore, escape metabolic inhibition by such reagents as deoxyspergualin, and can be stimulated to differentiate into antibody-secreting cells by subsequent encounter with antigen. One way to approach this problem is by hyperstimulation of cell surface anti-Gal $\alpha$ 1,3Gal antibody producing B cells. Hyperstimulation will result in the antigen-driven proliferation of these cells. Once these B cells enter the cell cycle and begin to undergo DNA

synthesis they will become sensitive to DNA alkylating agents, such as cyclophosphamide.

In order to test this hypothesis, the GalT<sup>-/-</sup> strain of mice, which has been genetically manipulated to eliminate the  $\alpha$ 1,3 galactosyltransferase gene activity, is used. These mice produce natural anti-Gal $\alpha$ 1,3Gal antibodies and can therefore be used as a small animal model for evaluating methods to the tolerize against the Gal $\alpha$ 1,3Gal epitopes.

The specific methodology was as follows:

Gal $\alpha$ 1,3Gal-BSA containing 17 Gal $\alpha$ 1,3GalB1,4G1cNAc structures per molecule (Alberta Research Council), 50 ug/300ml in 50% Complete Freund's Adjuvant/PBS, was injected intraperitoneally (i.p.) on day 0. Cyclophosphamide (Sigma), 200 mg/kg in 200 ul PBS, was injected i.p. as single bolus at one of the following times following Gal $\alpha$ 1,3Gal-BSA injection: 2 hr, 1 day, 2 days, 3 days, 4 days. Control mice received either cyclophosphamide alone or Gal $\alpha$ 1,3Gal-BSA alone. On day 7, i.e., one week following the Gal $\alpha$ 1,3Gal-BSA injection, mice were injected i.p. with  $10^7$  pig PBMC. Three mice were used for each time point of cyclophosphamide administration.

Sera were collected from the tail vein of GalT<sup>-/-</sup> or wild type mice on day 0 (prior to antigen injection), on day 7 (prior to challenge with pig PBMC); and on days 14 and 21 (i.e., 1 and 2 weeks following pig PBMC injection).

Anti-Gal $\alpha$ 1,3Gal versus anti-pig responses were measured, using flow cytometric analysis, see above, on sera collected on day 21.

Serum anti-Gal $\alpha$ 1,3Gal levels were quantified using an ELISA.

In order to inhibit the *in vivo* capacity of B cells to mature as anti-Gal $\alpha$ 1,3Gal antibody secretors, a protocol was devised in which B cells were first stimulated to enter mitosis by the presence of antigen binding to their cell surface immunoglobulin receptors. This antigenic stimulation was followed by inhibition of DNA synthesis by a single high dose of cyclophosphamide. Antigenic stimulation with Gal $\alpha$ 1,3Gal-BSA was followed by a time course of a single i.p. injection of cyclophosphamide in GalT(-/-) mice. Seven days following antigenic stimulation (day 7), mice were injected i.p. with pig PBMC and the ability of the mice to produce serum anti-Gal $\alpha$ 1,3Gal antibodies was measured by ELISA. The results of these studies indicate that cyclophosphamide administered 1-2 days, but not at 3 or 4 days, following antigenic stimulation can suppress both the anti-Gal $\alpha$ 1,3Gal IgM and IgG response to pig PBMC (Figure 3). To determine whether the inhibition of anti-Gal $\alpha$ 1,3Gal antibody production in response to pig PBMC was a consequence of the antigenic stimulation with Gal $\alpha$ 1,3Gal-BSA, the experiment was repeated but without the injection of Gal $\alpha$ 1,3Gal-BSA. In the absence of antigenic stimulation there was no inhibition of anti-Gal $\alpha$ 1,3Gal antibody production (Figure 4).

Serum samples taken two weeks after injection of the pig PBMC were analyzed for reactivity towards pig antigens by flow cytometry. In the mice given Gal $\alpha$ 1,3Gal-BSA, the fraction of anti-Gal $\alpha$ 1,3Gal IgM following pig PBMC stimulation, i.e., no CYP control, is approximately 80%; the fraction of anti-Gal $\alpha$ 1,3Gal IgG is very low (Figure 5). Analysis of the samples from the antigen-stimulated mice subsequently treated with cyclophosphamide on either day 1 or day

2 showed that the overall level of anti-pig IgM decreased and that the level of non-Gal $\alpha$ 1,3Gal IgM within the sample remained unchanged. This data indicate that (a) the decreased level of the anti-pig response reflects that, prior to antigenic stimulation, the majority of the B cells, are responsive to Gal $\alpha$ 1,3Gal and (b) the anti-Gal $\alpha$ 1,3Gal IgM response was specifically diminished. The sample taken from mice treated with cyclophosphamide on day 4, demonstrated that the fraction of anti-pig antibodies with Gal $\alpha$ 1,3Gal specificity was increased over the control, indicating that there had been active stimulation of the anti-Gal $\alpha$ 1,3Gal response.

In the animals treated with cyclophosphamide only (i.e., without Gal $\alpha$ 1,3Gal-BSA treatment) the subsequent injection of pig PBMC resulted in a IgM response which primarily directed towards the Gal $\alpha$ 1,3Gal moiety (Figure 6).

These experiments demonstrate that Gal $\alpha$ 1,3Gal-BSA stimulation followed by cyclophosphamide administration inhibits the ability of mice to produce anti-Gal $\alpha$ 1,3Gal antibodies in response to a potent immunogen, pig PBMC. The time window in which cyclophosphamide had its greatest effects on inhibition of anti-Gal $\alpha$ 1,3Gal antibody secretion suggests that B cell proliferation in response to antigenic stimulation is relatively rapid, as has been observed in other models. In a primed system, such as in the case of anti-Gal $\alpha$ 1,3Gal antibody production by GalT(-/-) mice, high responses to potent antigens are observed within one week of antigen exposure.

### Example 7

#### Effect of cyclophosphamide administration combined with $\alpha$ Gal-BSA pulses on maintenance of anti-Gal hyporesponsiveness

In order to inhibit the *in vivo* capacity of B cells to mature as anti-Gal secretors, a protocol was devised in which B cells were first stimulated to enter mitosis by the presence of antigen binding to their cell surface immunoglobulin receptors. This antigenic stimulation was followed by inhibition of DNA synthesis by a single high dose of cyclophosphamide.

Based on experimental models of tolerance induction in mice, it was predicted that the maintenance of hyporesponsiveness toward  $\alpha$ Gal epitopes would be dependent on a continuous source of antigen (i.e., Gal $\alpha$ 1,3Gal epitopes). Therefore, in the following experiments, the induction of an extended period of hyporesponsiveness in GalT(-/-) mice toward cells expressing  $\alpha$ Gal epitopes was demonstrated.

The specific methodology was as follows:

The administration of a high dose of  $\alpha$ Gal-BSA (500ug) was followed 40 hours later by cyclophosphamide (200mg/kg) injected i.p. and pulses of deaggregated  $\alpha$ Gal-BSA (50ug) every other day thereafter. Subsequent to these conditioning steps, sera were collected from these mice and control mice on a weekly basis and analyzed by an anti-Gal ELISA assay specific for mouse anti-Gal.

The results, as shown in Figure 7, represent the average anti-Gal production of four mice per data point.

These data demonstrated the antigenic pulses followed by metabolic inhibition with cyclophosphamide can diminish the sensitization toward  $\alpha$ Gal epitopes on porcine cells *in vivo*. It can be inferred from these data that the main effect of  $\alpha$ Gal-BSA pulses was to affect a class switch from IgM to IgG anti-Gal in the absence of the cyclophosphamide pulse. However, the cyclophosphamide pulse following antigenic stimulation resulted in an abrogation of the anti-Gal IgM response as well as a dampening of the anti-Gal IgG response to a pig PBMC challenge. It is expected that antigenic stimulation followed by cyclophosphamide and pulses of  $\alpha$ Gal-BSA, if repeated every two weeks, will lead to an abrogation of both IgM and IgG anti-Gal responses.

#### Example 8

##### Tolerization of a Gal $\alpha$ 1,3Gal deficient mouse to a Gal $\alpha$ 1,3Gal+ marrow graft

GalT-/- mice have been described by Thall *et al.*, *J. Biol. Chem.* 270: 21437-21440 (1995). These mice have a disrupted  $\alpha$ 1,3galactosyltransferase gene and lack the Gal $\alpha$ 1,3Gal epitope. They are capable of mounting an HAR response against wild type tissues. The mice are injected i.p. with a single dose (1 g per 25 kg body weight) of a conjugate prepared according to Example 4, followed by doses at 0.1 g per 25 kg every other day for eight days. Then, wild type murine bone marrow cells are implanted in the tail vein. The graft is expected to survive longer than in mock treated controls.



### Example 9

#### Tolerization to a porcine xenograft in a mouse model

Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA (17 haptens/BSA molecule) was purchased from the Alberta Research Council. This compound was injected i.p. into GalT $^{-/-}$  mice at 50 $\mu$ g/300ml in 50% complete Freund's adjuvant/saline. Cyclophosphamide (Sigma) was injected i.p. into the mice at 200 mg/kg in 200 $\mu$ l PBS as a single bolus 2 hours, 1 day, 2 days, 3 days and 4 days following injection of the Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA. Pig peripheral blood leukocytes were obtained from Charles River mini-swine by conventional Hypaque density gradient separation. One week after injection with the Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA, the mice were injected i.p. with 10 $^7$  pig peripheral blood leukocytes. Sera were collected from the mice before injection with the Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA, before challenge with the pig bone marrow cells, and at one and two weeks following challenge with pig bone marrow cells. Sera were diluted 1:20 into PBS and added in serial five-fold dilutions to microtiter wells coated with Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA. After incubation at 37°C for one hour, the wells were washed with PBS and a peroxidase substrate was added, followed by color development. Serum levels of antibody to Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA were quantified by OD measurement. Serum levels of both IgM and IgG specific for Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA were significantly lower than in control animals injected with Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA without subsequent cyclophosphamide treatment, or in control animals treated with cyclophosphamide without prior injection with Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA. These results demonstrate that stimulation with a

Gal $\alpha$ 1,3 epitope-containing immunogen, followed by treatment with a chemotherapeutic agent can induce tolerance to the Gal $\alpha$ 1,3 epitope.

#### Example 10

##### Tolerization of a primate to a porcine graft

Seventeen days prior to organ transplantation (day-17), adult baboons are set up with lines by exposure and cannulation of the aorta and vena cava using silastic shunts to create a loop. The lines run through a Synsorb 90 Gal $\alpha$ 1,3Gal affinity column (Alberta Research Council) prepared according the manufacturer's directions. During this procedure, the baboon is anesthetized with halothane and maintained by general endotracheal intubation anesthesia with monitoring of blood pressure, blood oxygen, blood gases and pH. The baboon's blood is perfused through the column for 60 minutes. The efficacy of this technique for removing antibodies specific for Gal $\alpha$ 1,3Gal epitope is measured by flow cytometry. The baboon's blood is perfused three blood volumes on each of days -15, i.e., -15 and -14. Splenectomy is performed on day -16. Tolerization of the baboon to the Gal $\alpha$ 1,3Gal epitope is achieved using any of the tolerogenic compounds, e.g., Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-BSA (see Examples 7 and 9). On days -3, -2, and -1, 50 mg/kg ATG is administered. On days -6 and -5, two doses of 150 whole body irradiation is administered. On day -1, 700 rads thymic irradiation is administered, pig bone marrow cells are administered (between  $1 \times 10^8$  and  $20 \times 10^8$  cells/kg); also, the baboon's heart or kidney is replaced with the heart or kidney of a Charles River SLA inbred miniswine, having the same swine MHC

haplotype as the donor bone marrow cells. Cyclosporin A is administered to maintain a blood level of 1,600 ng/ml. Daily administration of porcine cytokines IL-3, stem cell factor, and GM-CSF each at 100 ug/kg is initiated. These cytokines are produced as described in U.S. Patent No. 5,589,582, which is hereby incorporated by reference. Other treatments may include administration of deoxyspergualin or Mofetil.

### Example 11

#### Tolerization of a mouse using a

#### Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc/ceramide/sphingosine liposome

A baboon is treated exactly as described in Example 10, except that cyclophosphamide is not administered and the porcine bone marrow is replaced by the liposome (1mg/kg) prepared according to Example 3. Again, organ survival is expected to be better than for the mock-treated control.

### Example 12

#### Effect of $\alpha$ Gal and $\alpha$ Gal/DMS containing liposomes

#### *in vitro* and *in vivo* in mice on anti-Gal production

N,N-Dimethylsphingosine (DMS), a terminal component of the sphingosine metabolic pathway, has been shown by others to inhibit the proliferation of human B cell lines via apoptosis. To determine whether anti-Gal secreting cells from tissues were also susceptible to metabolic inhibition through the sphingosine cell death

pathway, these lymphocytes were removed from the spleen of GalT(-/-) mice. These cells were treated with DMS and  $\alpha$ Gal containing liposomes followed by an anti-Gal specific ELISPOT assay as described in Example 5.

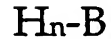
The specific methodology was as follows:

Blank liposomes were composed of phosphatidylcholine (PC) and cholesterol (C) (60:40). Alpha-Gal liposomes consisted of PC:C:Gal-HDPE (60:40:3). DMS = alphaGal liposomes consisted of PC:C:DMS:Gal-HDPE (60:40:6:3). Liposomes were prepared by drying compounds under vacuum, followed by rehydration and extrusion to produce 100nm liposomes in a Lipex extruder (Lipex Biomembranes, Vancouver, B.C.) Liposome size was confirmed on a particle size analyzer (Brookhaven Instruments, Holtsville, N.Y.)

Figure 8 shows that  $\alpha$ Gal and  $\alpha$ Gal/DMS-containing liposomes completely inhibit *in vitro*  $\alpha$ Gal production. These data demonstrated that liposomes incorporated with glycolipids containing either terminal  $\alpha$ galactosyl structures or N,N-Dimethylsphingosine are capable of down-modulating anti-Gal production.

What is claimed is:

1. A tolerogenic composition comprising the structure



wherein H represents a Gal $\alpha$ 1,3 Gal epitope-containing hapten, B represents a backbone and - represents an operable linkage, and wherein n is a number from 1 to 100,000,000.

2. The tolerogenic composition according to claim 1, wherein H has a terminal Gal $\alpha$ 1,3Gal epitope.
3. The tolerogenic composition according to claim 2, wherein H is a galactosyl( $\alpha$ 1,3)galactose disaccharide or trisaccharide.
4. The tolerogenic composition according to claim 3, wherein H is Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc or Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc.
5. The tolerogenic composition according to claim 1, wherein B is a non-immunogenic backbone.
6. The tolerogenic composition according to claim 1, wherein B is a non-immunogenic polymer having a molecular weight of from about 5,000 Da to about 100,000 Da.

7. The tolerogenic composition according to claim 6, wherein B is selected from polyvinylethanolamines, polyacrylamides, dextrans and polyglycomers.

8. The tolerogenic composition according to claim 7, wherein B is a polyvinylethanolamine in the molecular weight range of about from 10,000 to about 14,000 Da.

9. The tolerogenic composition according to claim 8, wherein n is 1-5.

10. The tolerogenic composition according to claim 1, wherein B is incorporated in a liposome.

11. The tolerogenic composition according to claim 7, wherein B is a polyacrylamide/amine in the molecular weight range of from about 40,000 to about 60,000 Da.

12. The tolerogenic composition according to claim 11, wherein n is from about 6 to about 25.

13. The tolerogenic composition according to claim 12, wherein n is from about 11 to 19.

14. The tolerogenic composition according to claim 7, wherein B is a dextran in the molecular weight range of from about 60,000 to about 100,000 Da.
15. The tolerogenic composition according to claim 14, wherein B is Dextran 70.
16. The tolerogenic composition according to claim 14, wherein n is from about 20 to about 40.
17. The tolerogenic composition according to claim 14, wherein n is about 30.
18. The tolerogenic composition according to claim 7, wherein B is a polyglycomer in the molecular weight range of from about 5,000 to about 100,000 Da.
19. The tolerogenic composition according to claim 18, wherein the polyglycomer contains divalent  $\text{Gal}\alpha 1,3\text{Gal}\beta\text{D1,4GlcNAc}$  or  $\text{Gal}\alpha 1,3\text{Gal}$  spaced at regular intervals.
20. The tolerogenic composition according to claim 18, wherein n is from about 20 to about 40.

21. The tolerogenic composition according to claim 20, wherein n is about 30.
22. The tolerogenic composition according to claim 1, wherein B is a non-immunogenic cellular antigen.
23. The tolerogenic composition according to claim 22, wherein B is an autologous, syngeneic, or xenogeneic erythrocyte.
24. The tolerogenic composition according to claim 23, wherein n is from about 1 to about 1,000.
25. A method for promoting in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, the method comprising the step of administering to the animal of the first species a tolerogenic amount of a tolerogenic composition according to claim 1.
26. The method according to claim 25, wherein the animal of the first animal species is a human and the animal of the second animal species is a pig.



27. A tolerogenic composition comprising the structure



wherein H is a Gal $\alpha$ 1,3 Gal epitope-containing hapten, T is an agent which is cytotoxic to or modulates the function of T and B cells and - represents an operable linkage, and wherein n is a number from 1 to 1,000.

28. The tolerogenic composition according to claim 27, wherein H has a terminal Gal $\alpha$ 1,3Gal epitope.

29. The tolerogenic composition according to claim 28, wherein H is a galactosyl( $\alpha$ 1,3)galactose dissacharide or trisaccharide.

30. The tolerogenic composition according to claim 29, wherein H is Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc or Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc.

31. The tolerogenic composition according to claim 27, wherein T is selected from a ceramide, a lipophilic toxin, a radioisotope, diptheria toxin, ricin A chain, saponin, and doxorubicin.

32. The tolerogenic composition according to claim 31, wherein T is ceramide pentahehexoside.

33. The tolerogenic composition according to claim 31, wherein the lipophilic toxin is selected from DMS, TMS and ceramide analogs.
34. The tolerogenic composition according to claim 31, wherein the radioisotope is selected from a radioisotope of Tc,  $^{131}\text{I}$  and  $^{90}\text{Yt}$ .
35. The tolerogenic composition according to claim 27, wherein the operable linkage is a covalent linkage directly between H and T.
36. The tolerogenic composition according to claim 27, wherein H and T are bound to a carrier molecule.
37. The tolerogenic composition according to claim 36, wherein the carrier molecule is selected from a neoglycoprotein, a glycoprotein, a peptide, a glycolipid, autologous IgG, or a synthetic carrier.
38. The tolerogenic composition according to claim 37, wherein the neoglycoprotein is bovine serum albumin or human serum albumin.
39. The tolerogenic composition according to claim 37, wherein the glycolipid is selected from HDPE, ceramide and ceramide analogs.

40. The tolerogenic composition according to claim 37, wherein the synthetic carrier is syalylacNAc-HDPE.
41. The tolerogenic composition according to claim 27, wherein the operable linkage is a lipophilic association.
42. The tolerogenic composition according to claim 41, wherein the lipophilic association forms a liposome.
43. The tolerogenic composition according to claim 42, wherein the liposome contains T and in which H is covalently linked to a lipophilic molecule associated with the liposome.
44. The tolerogenic composition according to claim 43, wherein the lipophilic molecule is selected from phosphatidylcholine, cholesterol, phosphatidylethanolamine, and synthetic neoglycolipids.
45. The tolerogenic composition according to claim 44, wherein the synthetic neoglycolipid is syalylacNAc-HDPE.
46. The tolerogenic composition according to claim 45, wherein T is a ceramide.

47. The tolerogenic composition according to claim 46, wherein T is ceramide pentahexoside.
48. The tolerogenic composition according to claim 47, further comprising an inhibitor of PKC or Bcl-2.
49. The tolerogenic composition according to claim 48, wherein the PKC or Bcl-2 inhibitor is a sphingosine.
50. The tolerogenic composition according to claim 49, wherein the sphingosine is N,N-dimethylsphingosine or N,N,N-trimethylsphingosine (TMS).
51. The tolerogenic composition according to claim 27, further being operably linked to a T cell receptor binding peptide or protein.
52. A method for promoting in a an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, the method comprising administering to the animal of the first species a tolerogenic amount of a tolerogenic composition according to claim 26.
53. The method according to claim 52, wherein the animal of the first animal species is a human and the animal of the second animal species is a pig.

54. A method for promoting in an animal of a first animal species a state of tolerance against Gal $\alpha$ 1,3Gal epitopes present on a xenograft from an animal of a second animal species, the method comprising administering to the animal of the first species an immunogenic composition comprising the structure



wherein H represents a Gal $\alpha$ 1,3 Gal epitope-containing hapten as described for the first aspect of the invention, I represents an immunogenic carrier and - represents an operable linkage, and wherein n is a number from 1 to 100,000,000, then treating the animal with a chemotherapeutic agent.

55. The method according to claim 54, wherein the animal of the first animal species is a human and the animal of the second animal species is a pig.

56. The method according to claim 54, wherein H has a terminal Gal $\alpha$ 1,3Gal epitope.

57. The method according to claim 56, wherein H is a galactosyl( $\alpha$ 1,3)galactose disaccharide or trisaccharide.

58. The method according to claim 57, wherein H is Gal $\alpha$ 1,3Gal $\beta$ 1,4Glc or Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc.

59. The method according to claim 54, wherein I is selected from immunogenic proteins, immunogenic lipids, and immunogenic carbohydrates.
60. The method according to claim 59, wherein the immunogenic protein is selected from BSA, KLH and cholera toxin B subunit.
61. The method according to claim 59, wherein the immunogenic lipid is a rabbit erythrocyte membrane.
62. The method according to claim 59, wherein the immunogenic carbohydrate is a polymer with a molecular weight greater than 100,000 Da.
63. The method according to claim 54, wherein the chemotherapeutic agent is selected from cyclophosphamide, leflunomide, mycophenolate, mofetil, doxorubicin, 2,3-dideoxyinosine, chlorambucil, steroid hormones, adriamycin, brequinar analogs, and melphalan.
64. The method according to claim 54, wherein the immunogenic composition is administered in the presence of adjuvant.
65. The method according to claim 64, wherein the adjuvant is selected from alum, RIBI and Freund's complete adjuvant.

66. The method according to claim 54, wherein the immunogenic composition is administered in the presence of xenogeneic lymphocytes or bone marrow cells.

67. The method according to claim 66, wherein the xenogeneic lymphocytes or bone marrow cells are from an animal of the second animal species.

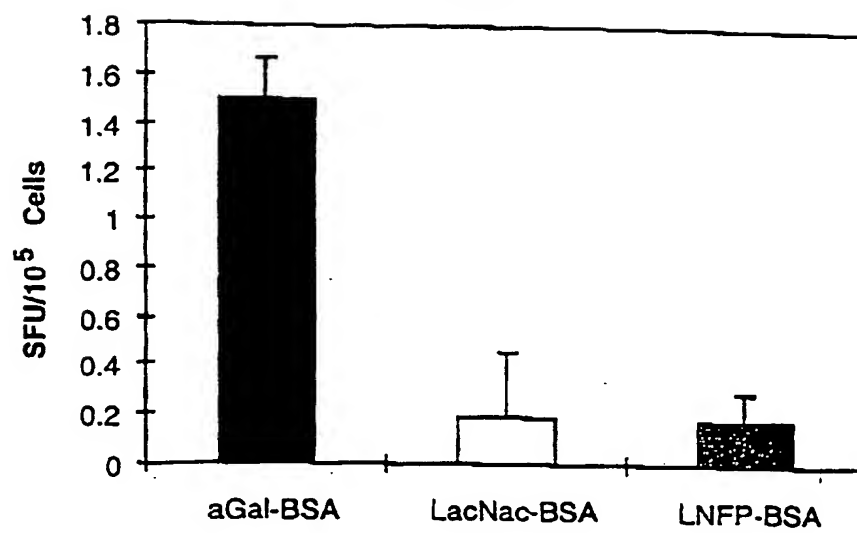
68. A method for determining the frequency of  $\alpha$ Gal secreting B cells, wherein the method comprises performing an ELISPOT assay.

69. The method according to claim 68, wherein the ELISPOT assay comprises the steps of

- a. isolating lymphocytes or lymphocyte preparations enriched for B cells;
- b. incubating the isolated cells in a microwell in a hybridoma culture medium;
- c. transferring the cells to nitrocellulose bottom microtiter wells coated with antigen;
- d. incubating the cells overnight in the microtiter wells;
- e. washing the cells from the walls of the microtiter wells;
- f. adding goat anti-mouse IgM or IgG conjugated horseradish peroxidase to the microtiter wells;
- g. adding peroxidase substrate to the microtiter wells;
- h. counting the spot formations in the microtiter wells as clonal secretion of antigen specific antibody; and
- i. determining the frequency of cells secreting antibody per  $10^5$  added to the microtiter wells.

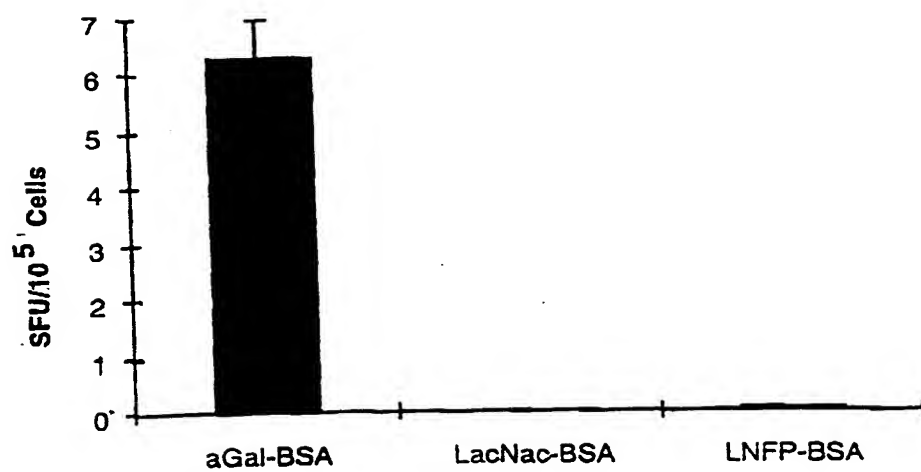
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FIGURE 1



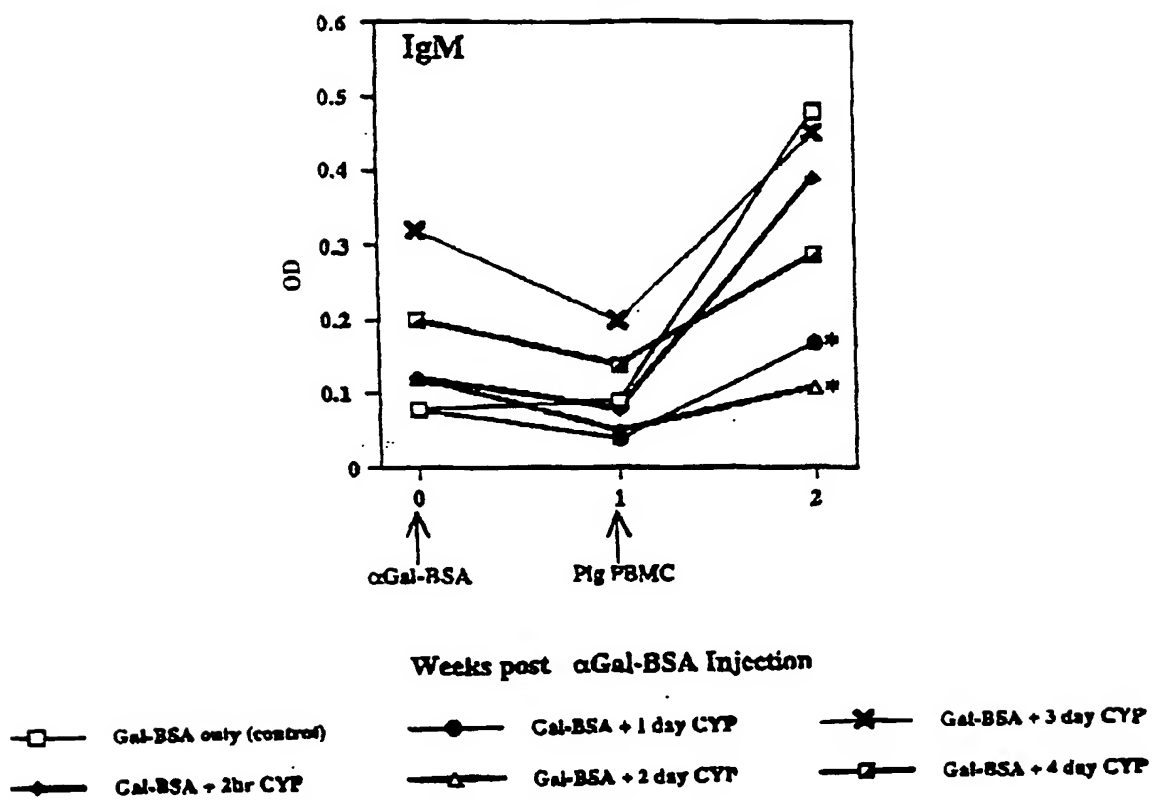


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FIGURE 2

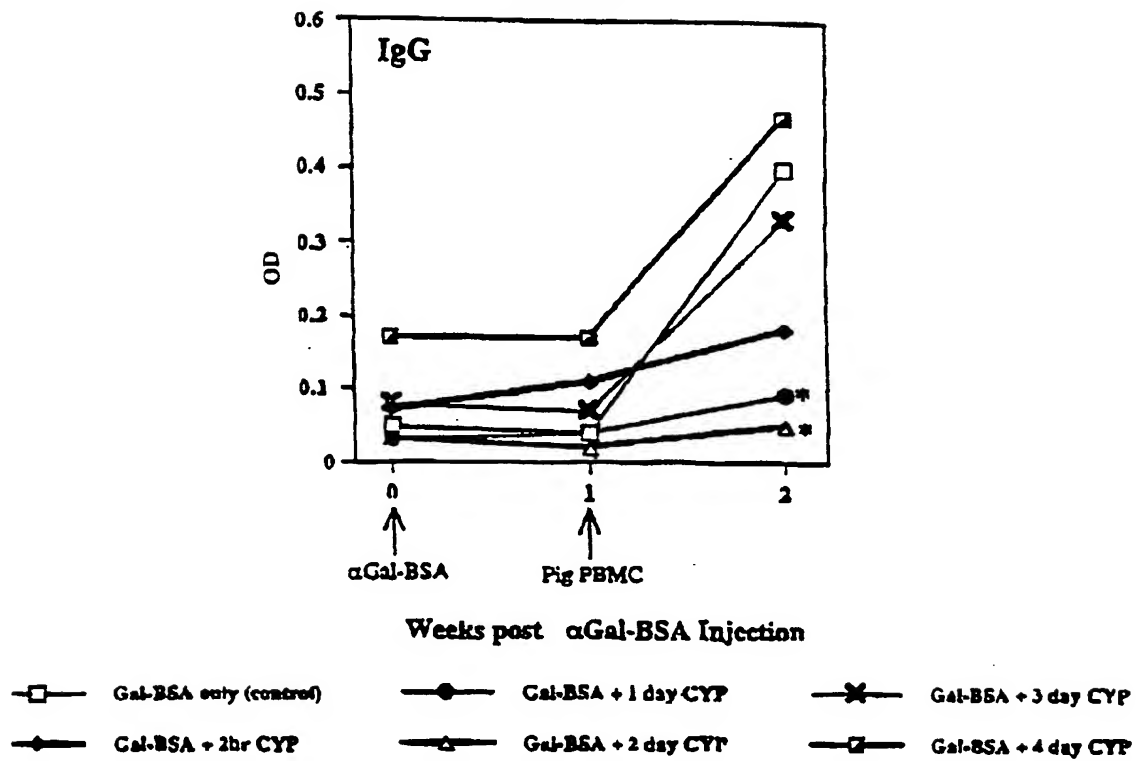
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FIGURE 3A



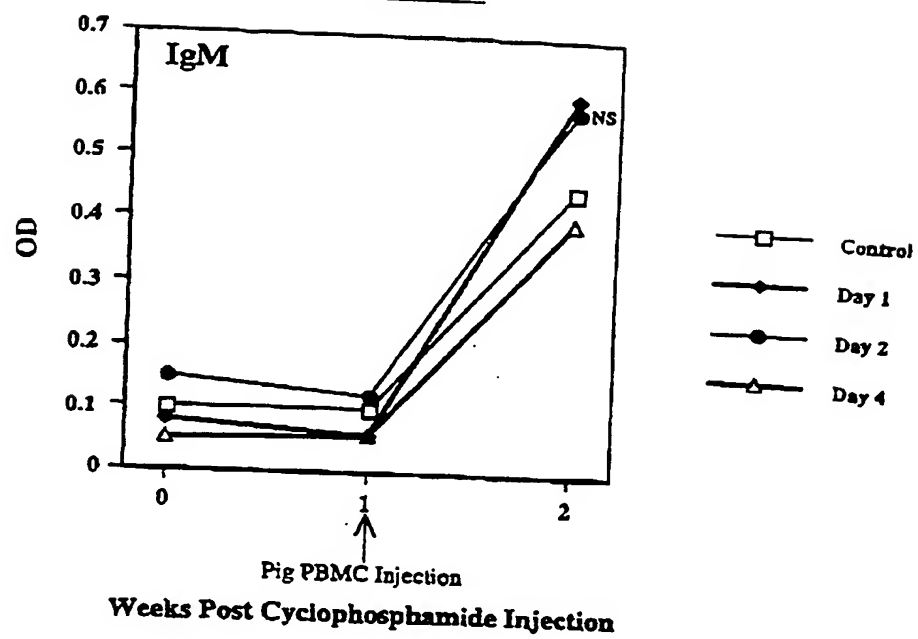
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FIGURE 3B



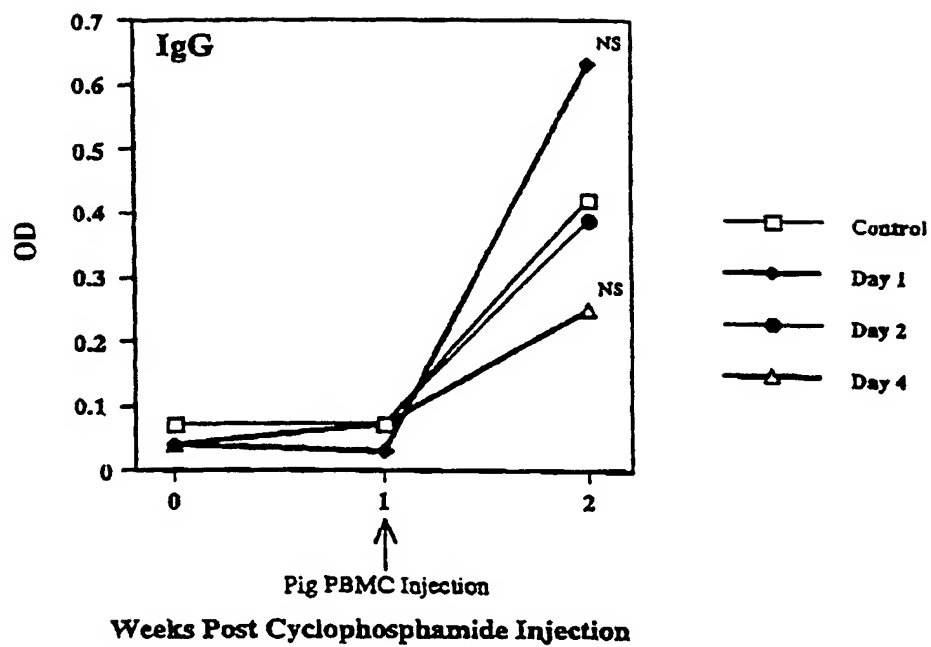
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FIGURE 4A



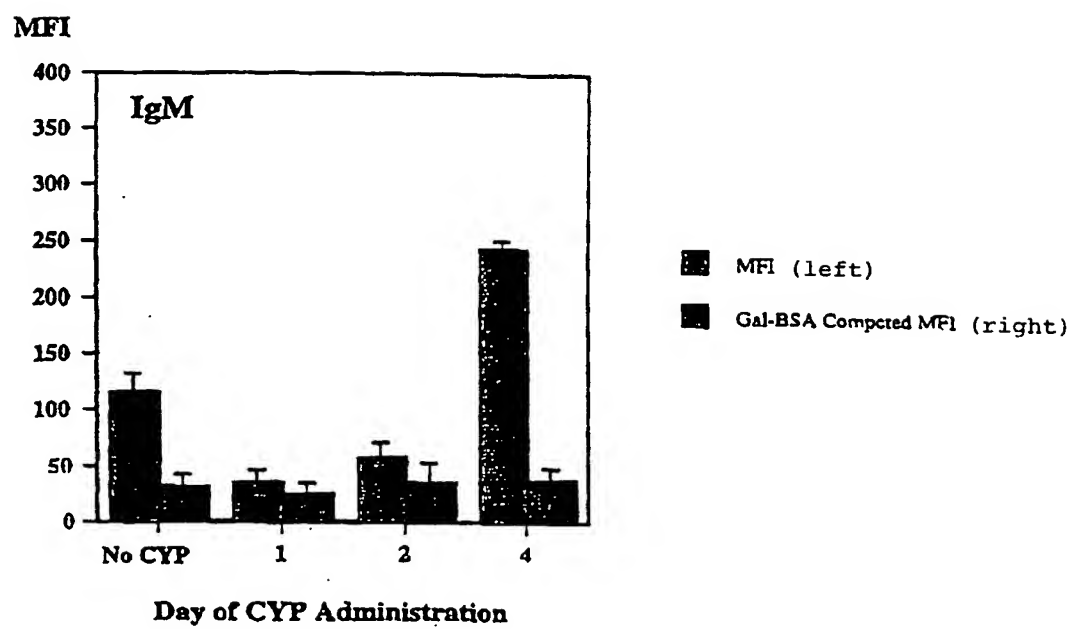
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FIGURE 4B



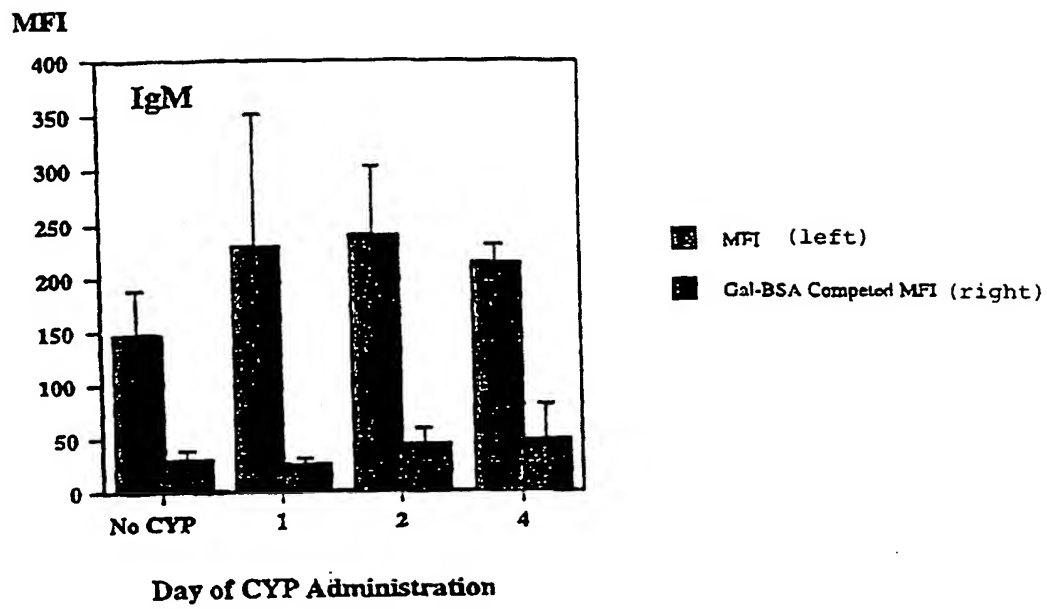
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FIGURE 5



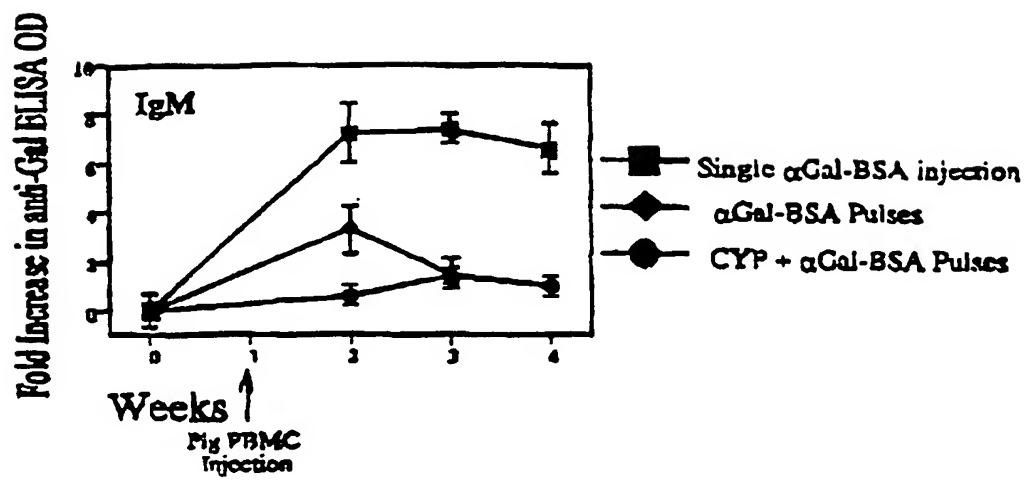
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FIGURE 6



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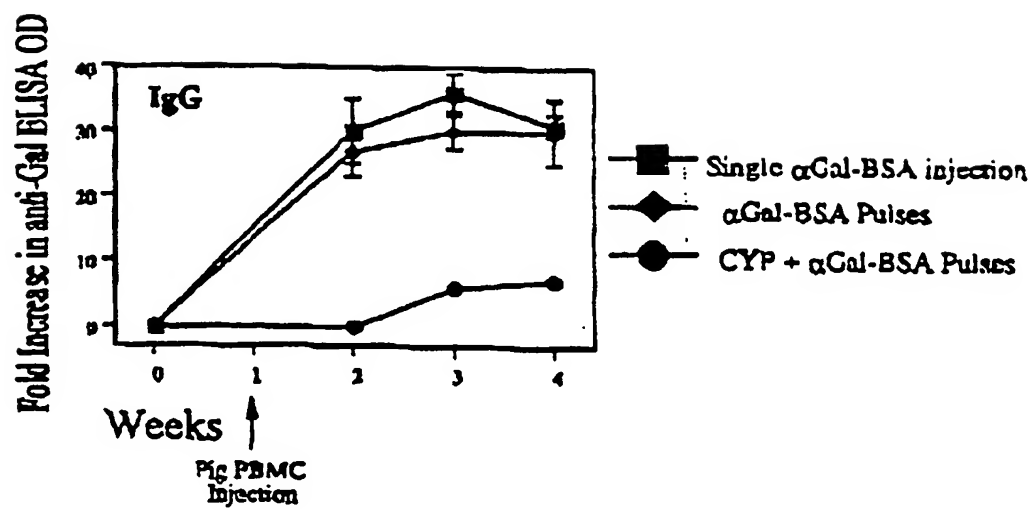
FIGURE 7A





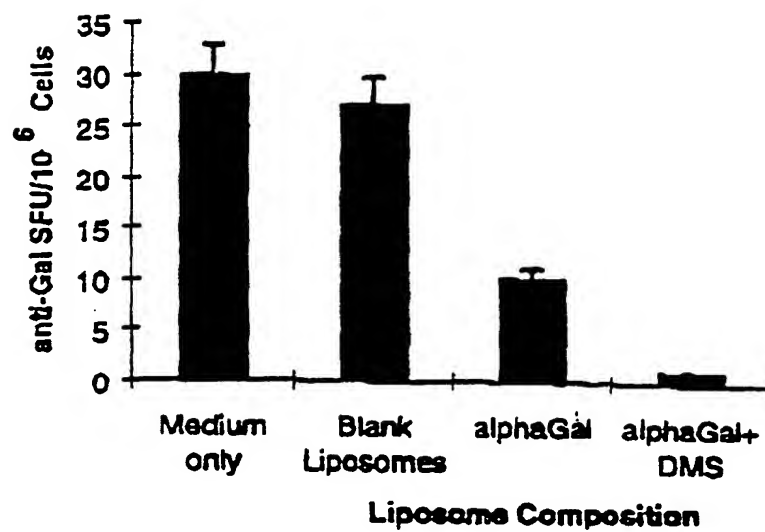
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FIGURE 7B



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FIGURE 8



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